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(54) Title: TRANSFORMED EUKARYOTIC CELLS, AND TRANSPOSON-BASED TRANSFORMATION VECTORS (57) Abstract Methods are disclosed for production of natural or synthetic lytic peptides in transformed eukaryotic cells. The lytic peptides include cecropins, melittins, defensins, magainins, sarcotoxins, and analogs of said peptides. Vectors for expressing said peptides in transformed cells are disclosed, as are transformed fetal donkey dermal cells and transgenic catfish. Said transformation increases the host cells' resistance to bacterial pathogens. The moth cecropin B promoter is shown to function in mammalian cells. A transposon-based vector which enhances DNA integration into eukaryotic host genomes is disclosed.		

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Transformed Eukaryotic Cells, and Transposon-Based Transformation Vectors

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This invention pertains to transformed animals and animal cells capable of expressing exogenous lytic peptides; and to genes in eukaryotic cells controlled by exogenous promoters that are responsive to inducers of acute phase proteins; and to transposon-based transformation vectors.

BACKGROUND ART

Lytic Peptides

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Few effective treatments exist for either acute or chronic intracellular bacterial, protozoal, or viral diseases of animals, including humans. In many such infections, the infectious agent is localized within host cells. Due to the intracellular location of the infectious agents, the host immune system is often ineffective. Likewise, anti-pathogenic compounds are often ineffective, due to their difficulty in crossing host cell membranes.

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Beck *et al.*, "Invertebrate Cytokines III: Invertebrate Interleukin-1-like Molecules Stimulate Phagocytosis by Tunicate and Echinoderm Cells," Cellular Immunology, vol. 146, pp. 284-299 (1993) discusses relationships among phagocytotic mechanisms of different phyla.

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Lytic peptides are small, basic proteins. Native lytic peptides appear to be major components of the antimicrobial defense systems of a number of animal species, including those of insects, amphibians, and mammals. They typically comprise 23-39 amino acids, and have the potential for forming amphipathic alpha-helices. See Boman *et al.*, "Humoral immunity in *Cecropia* pupae," Curr. Top. Microbiol. Immunol. vol. 94/95, pp. 75-91 (1981); Boman *et al.*, "Cell-free immunity in insects," Annu. Rev. Microbiol., vol. 41, pp. 103-126 (1987); Zasloff, "Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial DNA sequence of a precursor," Proc. Natl. Acad. Sci. USA, vol. 84, pp. 3628-3632 (1987); Ganz *et al.*, "Defensins natural peptide antibiotics of human neutrophils," J. Clin. Invest., vol. 76, pp. 1427-1435 (1985); and Lee *et al.*, "Antibacterial peptides from pig intestine: isolation of a mammalian cecropin," Proc. Natl. Acad. Sci. USA, vol. 86, pp. 9159-9162 (1989).

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Known amino acid sequences for lytic peptides may be modified to create new peptides that would also be expected to have lytic activity by substitutions of amino acid residues that preserve the amphipathic nature of the peptides (e.g., replacing a polar

residue with another polar residue, or a non-polar residue with another non-polar residue, etc.); by substitutions that preserve the charge distribution (e.g., replacing an acidic residue with another acidic residue, or a basic residue with another basic residue, etc.); or by lengthening or shortening the amino acid sequence while preserving its amphipathic character or its charge distribution. Lytic peptides and their sequences are disclosed in Yamada *et al.*, "Production of recombinant sarcotoxin IA in *Bombyx mori* cells," Biochem. J., Vol. 272, pp. 633-666 (1990); Tanai *et al.*, "Isolation and nucleotide sequence of cecropin B cDNA clones from the silkworm, *Bombyx mori*," Biochimica Et Biophysica Acta, Vol. 1132, pp. 203-206 (1992); Boman *et al.*, "Antibacterial and antimalarial properties of peptides that are cecropin-melittin hybrids," Febs Letters, Vol. 259, pp. 103-106 (1989); Tessier *et al.*, "Enhanced secretion from insect cells of a foreign protein fused to the honeybee melittin signal peptide," Gene, Vol. 98, pp. 177-183 (1991); Blondelle *et al.*, "Hemolytic and antimicrobial activities of the twenty-four individual omission analogs of melittin," Biochemistry, Vol. 30, pp. 4671-4678 (1991); Andreu *et al.*, "Shortened cecropin A-melittin hybrids. Significant size reduction retains potent antibiotic activity," Febs Letters, Vol. 296, pp. 190-194 (1992); Macias *et al.*, "Bactericidal activity of magainin 2: use of lipopolysaccharide mutants," Can. J. Microbiol., Vol. 36, pp. 582-584 (1990); Rana *et al.*, "Interactions between magainin-2 and *Salmonella typhimurium* outer membranes: effect of Lipopolysaccharide structure," Biochemistry, Vol. 30, pp. 5858-5866 (1991); Diamond *et al.*, "Airway epithelial cells are the site of expression of a mammalian antimicrobial peptide gene," Proc. Natl. Acad. Sci. USA, Vol. 90, pp. 4596 ff (1993); Selsted *et al.*, "Purification, primary structures and antibacterial activities of β -defensins, a new family of antimicrobial peptides from bovine neutrophils," J. Biol. Chem., Vol. 268, pp. 6641 ff (1993); Tang *et al.*, "Characterization of the disulfide motif in BNBD-12, an antimicrobial β -defensin peptide from bovine neutrophils," J. Biol. Chem., Vol. 268, pp. 6649 ff (1993); Lehrer *et al.*, Blood, Vol. 76, pp. 2169-2181 (1990); Ganz *et al.*, Sem. Resp. Infect. I., pp. 107-117 (1986); Kagan *et al.*, Proc. Natl. Acad. Sci. USA, Vol. 87, pp. 210-214 (1990); Wade *et al.*, Proc. Natl. Acad. Sci. USA, Vol. 87, pp. 4761-4765 (1990); and Romeo *et al.*, J. Biol. Chem., Vol. 263, pp. 9573-9575 (1988).

Lytic peptides typically have a broad spectrum of activity (e.g., against gram negative bacteria, fungi, protozoa, and viruses). Their activity is both direct and indirect (e.g., virus-infected cells are destroyed, disrupting virus production). Thus some pathogens that have developed the ability to avoid host defenses are nevertheless susceptible to destruction by lytic peptides.

At least four families of naturally-occurring lytic peptides have been discovered in the last decade: cecropins, defensins, sarcotoxins, and magainins. Boman and coworkers in Sweden performed the original work on the humoral defense system of *Hyalophora cecropia*, the giant silk moth, to protect itself from bacterial infection. See Hultmark *et al.*, "Insect immunity. Purification of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*," Eur. J. Biochem., vol. 106, pp. 7-16 (1980); and Hultmark *et al.*, "Insect immunity. Isolation and structure of cecropin D. and four minor antibacterial components from *cecropia* pupae," Eur. J. Biochem., vol. 127, pp. 207-217 (1982).

Infection in *H. cecropia* induces the synthesis of specialized proteins capable of disrupting bacterial cell membranes, resulting in lysis and cell death. Among these specialized proteins are those known collectively as cecropins. The principal cecropins -- cecropin A, cecropin B, and cecropin D -- are small, highly homologous, basic peptides. In collaboration with Merrifield, Boman's group showed that the amino-terminal half of the various cecropins contains a sequence that will form an amphipathic alpha-helix. Andreu *et al.*, "N-terminal analogues of cecropin A: synthesis, antibacterial activity, and conformational properties," Biochem., vol. 24, pp. 1683-1688 (1985). The carboxy-terminal half of the peptide comprises a hydrophobic tail. See also Boman *et al.*, "Cell-free immunity in *Cecropia*," Eur. J. Biochem., vol. 201, pp. 23-31 (1991).

Recently, a cecropin-like peptide has been isolated from porcine intestine. Lee *et al.*, "Antibacterial peptides from pig intestine: isolation of a mammalian cecropin," Proc. Natl. Acad. Sci. USA, vol. 86, pp. 9159-9162 (1989).

Cecropin peptides have been observed to kill a number of animal pathogens other than bacteria. See Jaynes *et al.*, "In Vitro Cytocidal Effect of Novel Lytic Peptides on *Plasmodium falciparum* and *Trypanosoma cruzi*," FASEB, 2878-2883 (1988); Arrowood *et al.*, "Hemolytic properties of lytic peptides active against the sporozoites of *Cryptosporidium parvum*," J. Protozool., vol. 38, No. 6, pp. 161S-163S (1991); and Arrowood *et al.*, "In vitro activities of lytic peptides against the sporozoites of *Cryptosporidium parvum*," Antimicrob. Agents Chemother., vol. 35, pp. 224-227 (1991). However, normal mammalian cells do not appear to be adversely affected by cecropins, even at high concentrations. See Jaynes *et al.*, "In vitro effect of lytic peptides on normal and transformed mammalian cell lines," Peptide Research, vol. 2, No. 2, pp. 1-5 (1989); and Reed *et al.*, "Enhanced in vitro growth of murine fibroblast cells and preimplantation embryos cultured in medium supplemented with an amphipathic peptide," Mol. Reprod. Devel., vol. 31, No. 2, pp. 106-113 (1992).

Defensins, originally found in mammals, are small peptides containing six to eight cysteine residues. Ganz *et al.*, "Defensins natural peptide antibiotics of human neutrophils," J. Clin. Invest., vol. 76, pp. 1427-1435 (1985). Extracts from normal human neutrophils contain three defensin peptides: human neutrophil peptides HNP-1, HNP-2, and HNP-3. Defensin peptides have also been described in insects and higher plants. Dimarcq *et al.*, "Insect immunity: expression of the two major inducible antibacterial peptides, defensin and dipterecin, in *Phormia terranova*," EMBO J., vol. 9, pp. 2507-2515 (1990); Fisher *et al.*, Proc. Natl. Acad. Sci. USA, vol. 84, pp. 3628-3632 (1987).

Slightly larger peptides called sarcotoxins have been purified from the fleshfly *Sarcophaga peregrina*. Okada *et al.*, "Primary structure of sarcotoxin I, an antibacterial protein induced in the hemolymph of *Sarcophaga peregrina* (flesh fly) larvae," J. Biol. Chem., vol. 260, pp. 7174-7177 (1985). Although highly divergent from the cecropins and defensins, the sarcotoxins presumably have a similar antibiotic function.

Other lytic peptides have recently been found in amphibians. Gibson and collaborators isolated two peptides from the African clawed frog, *Xenopus laevis*, peptides which they named PGS and Gly¹⁰Lys²²PGS. Gibson *et al.*, "Novel peptide fragments originating from PGL₂ and the caervlein and xenopsin precursors from *Xenopus laevis*," J. Biol. Chem., vol. 261, pp. 5341-5349 (1986); and Givannini *et al.*, "Biosynthesis and degradation of peptides derived from *Xenopus laevis* prohormones," Biochem. J., vol. 243, pp. 113-120 (1987). Zasloff showed that the *Xenopus*-derived peptides have antimicrobial activity, and renamed them magainins. Zasloff, "Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial DNA sequence of a precursor," Proc. Natl. Acad. Sci. USA, vol. 84, pp. 3628-3632 (1987).

Synthesis of nonhomologous analogs of different classes of lytic peptides has revealed that a positively charged, amphipathic sequence containing at least 20 amino acids appears to be a requirement for lytic activity. Shiba *et al.*, "Structure-activity relationship of Lepidopteran, a self-defense peptide of *Bombyx mori*," Tetrahedron, vol. 44, No. 3, pp. 787-803 (1988); and unpublished data from our laboratory. A 20-mer appears to possess roughly the minimum alpha-helix length needed to span a cell membrane. Smaller peptides (or lower concentrations of peptide) not only fail to kill cells, but actually stimulate cell growth. Reed *et al.*, "Enhanced in vitro growth of murine fibroblast cells and preimplantation embryos cultured in medium supplemented with an amphipathic peptide," Mol. Reprod. Devel., vol. 31, No. 2, pp. 106-113 (1992); and unpublished data from our laboratory.

Cecropins have been shown to target pathogens or compromised cells selectively, without affecting normal host cells. The synthetic lytic peptide known as Shiva 1 has been shown to destroy intracellular *Brucella abortus*-, *Trypanosoma cruzi*-, *Cryptosporidium parvum*-, and infectious bovine herpes virus I (IBR)-infected host cells, with little or no toxic effects on noninfected mammalian cells. See Jaynes *et al.*, "In vitro effect of lytic peptides on normal and transformed mammalian cell lines," Peptide Research, vol. 2, No. 2, pp. 1-5 (1989); Wood *et al.*, "Toxicity of a Novel Antimicrobial Agent to Cattle and Hamster cells *In vitro*," Proc. Ann. Amer. Soc. Anim. Sci., Utah State University, Logan, UT. J. Anim. Sci. (Suppl. 1), vol. 65, p. 380 (1987); Arrowood *et al.*, "Hemolytic properties of lytic peptides active against the sporozoites of *Cryptosporidium parvum*," J. Protozool., vol. 38, No. 6, pp. 161S-163S (1991); Arrowood *et al.*, "In vitro activities of lytic peptides against the sporozoites of *Cryptosporidium parvum*," Antimicrob. Agents Chemother., vol. 35, pp. 224-227 (1991); and Reed *et al.*, "Enhanced in vitro growth of murine fibroblast cells and preimplantation embryos cultured in medium supplemented with an amphipathic peptide," Mol. Reprod. Devel., vol. 31, No. 2, pp. 106-113 (1992).

Also of interest are the following commonly-assigned patent applications: Jaynes *et al.*, "Method for Introduction of Disease and Pest Resistance Into Plants and Novel Genes Incorporated Into Plants Which Code Therefor," United States patent application S.N. 07/373,623, filed June 29, 1989; Jaynes *et al.*, "Plants Genetically Enhanced for Disease Resistance," United States patent application S.N. 07/646,449, filed January 25, 1991; Jaynes *et al.*, "Therapeutic Antimicrobial Polypeptides, Their Use and Methods for Preparation," S.N. 07/069,653, filed July 6, 1987; Jaynes *et al.*, "Inhibition of Eucaryotic Pathogens and Neoplasms and Stimulation of Fibroblasts and Lymphocytes with Lytic Peptides," United States patent application S.N. 07/102,175, filed September 29, 1987; and Jaynes, "Lytic Peptides: Their Use in the Treatment of Microbial Infections, Cancer and in the Promotion of Growth," United States patent application S.N. 07/336,181, filed April 10, 1989.

It is believed (without wishing to be bound by this theory) that lytic peptides act by disrupting cell membranes, and that normal host cells protect themselves through their ability to repair the resulting membrane damage. By contrast, bacteria, protozoa, and compromised host cells are unable (or less able) to repair damaged membranes. Because parasitized cells have a diminished capacity to repair membranes, after a lytic peptide "attack" they are preferentially destroyed, while adjacent normal cells repair their membranes and survive.

At least three modes have been proposed for the lytic peptide-membrane interaction that leads to cytolysis: (1) The amphipathic helix is located on the membrane surface, and the presence of the helix in the head group region disorders the lipid bilayer. Dawson *et al.*, "The interaction of bee melittin with lipid bilayer membranes," Biochem. Biophys. Acta., vol. 510, pp. 75-86 (1978). (2) Peptide oligomers form ion channels in the membrane, resulting in osmotically-induced lysis. Tosteson *et al.*, "The sting - melittin forms channels in lipid bilayers," Biophys. J., vol. 36, pp. 109-116 (1981). (3) The lytic peptide causes aggregation of native membrane proteins, resulting in the formation of channels or pores. Burt *et al.*, "Role of membrane proteins in monosodium urate crystal-membrane interactions. I. Effect of pretreatment of erythrocyte neuraminidase," J. Rheumatol., vol. 17, pp. 1353-1358 (1990).

Many intracellular obligate pathogens live inside host cells because they are vulnerable to host defenses when outside the cell, where they may be destroyed by humoral or cellular defenses, or by conventional therapeutic agents. Also, known viruses and intracellular protozoa require a staging development within a host cell before becoming infectious; if released prematurely they will not be infective. Evidence indicates that the released infective stages in bacterial, fungal, and protozoal pathogens are directly destroyed by lytic peptides. See Jaynes *et al.*, "In vitro effect of lytic peptides on normal and transformed mammalian cell lines," Peptide Research, vol. 2, No. 2, pp. 1-5 (1989); Jaynes *et al.*, "In Vitro Cytocidal Effect of Novel Lytic Peptides on *Plasmodium falciparum* and *Trypanosoma cruzi*," FASEB, 2878-2883 (1988); Arrowood *et al.*, "Hemolytic properties of lytic peptides active against the sporozoites of *Cryptosporidium parvum*," J. Protozool., vol. 38, No. 6, pp. 161S-163S (1991); and Arrowood *et al.*, "In vitro activities of lytic peptides against the sporozoites of *Cryptosporidium parvum*," Antimicrob. Agents Chemother., vol. 35, pp. 224-227 (1991).

Plants Transformed with Lytic Peptide Genes

A number of synthetic lytic peptides have been synthesized, retaining some properties of native lytic peptides. For example, Shiva I was designed as a substitution analog of native Cecropin B, having 46% homology to the natural Cecropin B molecule. However, the hydrophobic properties and charge density of the native structure were conserved in the synthetic peptide. Data supporting the ability of the Shiva I gene to enhance disease resistance has been obtained from transgenic plants. Genes encoding synthetic lytic peptides were chemically synthesized and cloned into the binary vector pBI121. For the less active peptide SB-37 (a cecropin analog), expression was controlled by a constitutive promoter, the 35S cauliflower mosaic virus 5' region-nopaline synthetase-3' polyadenylation cassette (Rogers *et al.*, "Improved vectors for plant

transformation: expression cassette vectors and new selectable markers," Meth. Enz. vol. 153, pp. 253-305 (1987)). For the more active Shiva I, expression was controlled by the wound-inducible plant promoter for proteinase inhibitor II ("Pill") (Sanchez-Serrano *et al.*, "Wound-induced expression of a potato proteinase inhibitor II gene in transgenic tobacco plants," EMBO J. vol. 6, pp. 303-306 (1987); Jaynes *et al.*, "Expression of a cecropin B lytic peptide analog in transgenic tobacco confers enhanced resistance to bacterial wilt caused by *Pseudomonas solanacearum*," Plant Science (1993).

In non-wounded potato plants, Pill accumulates in the tubers, with non-detectable levels of the protein in leaves, stem or roots. When the leaves are wounded, however, expression of the gene is induced not only in the wounded leaves, but also in non-wounded upper and lower leaves and in the upper part of the stem. Pena-Cortes *et al.*, "Systemic induction of proteinase-inhibitor-II gene expression into potato plants by wounding," Planta, vol. 174, pp. 84-91 (1988).

Transgenic tobacco plants with genes coding for lytic peptides have also been obtained via *Agrobacterium* transformation. Bioassays testing the disease resistance of F₁ progeny indicated that, compared to transgenic controls and SB-37 plants, Shiva-containing tobacco seedlings exhibited delayed wilt symptoms and reduced disease severity and mortality after infection with a highly virulent strain of *Pseudomonas solanacearum*. (*P. solanacearum* is a vascular pathogen that causes severe wilting.) Jaynes *et al.*, "Expression of a cecropin B lytic peptide analog in transgenic tobacco confers enhanced resistance to bacterial wilt caused by *Pseudomonas solanacearum*," Plant Science (1993). No enhanced resistance was observed for the plants producing the synthetic peptide SB-37, presumably because of its low bioactivity against this pathogen. Destefano-Beltran *et al.*, Mol. Biol. of the Potato, pp. 205-221 (1990).

In contrast to this work in plants, to the knowledge of the inventors, no previous work has resulted in the successful expression of exogenous cecropin (or any other lytic peptide) in mammalian or other animal cells. In fact, very few invertebrate genes have ever been stably expressed in a vertebrate cell, and even fewer non-mammalian genes have ever been stably expressed in a mammalian cell.

Transformation of Eukaryotic Genomes

A major problem in transforming DNA into eukaryotic cells, especially into the cells of mammals and other vertebrates, is the stable integration of the exogenous DNA into a recipient chromosome. Several techniques are currently used for the delivery of the DNA into the recipient host, techniques that have shown varying degrees of success. The first, and currently most common, method for transformation of animals is the microinjection of exogenous DNA into a one- or two-cell embryo. This procedure has

several drawbacks, including the following difficulties: (1) the technique requires a level of skill that is not available in most laboratories; (2) the procedure is very time-consuming, often requiring an entire day to microinject a few hundred embryos; and (3) the method has a relatively low success rate – typically about 1-3% of the injected embryos are observed to have a stable insertion.

The second most common procedure is electroporation. Electroporation has advantages over microinjection, primarily in speed. Using electroporation, several thousand embryos can be transformed in a day. Major limitations include the following: (1) the availability of embryos, (2) means to maintain and screen the embryos, and (3) the approximately 50% lethality levels caused by the electric currents typically used. The high lethality is somewhat offset by the increased number of embryos demonstrating positive expression, a number that can approach 15%, about five times that of the best microinjection rates.

A third method, currently gaining popularity, is the use of lipofection to deliver DNA packaged inside liposomes. Lipofection has the advantage over electroporation that it is not as lethal to the cells. Lipofection typically results in a 1-2% increase in successful transformations as compared to electroporation.

However, each of these three transformation methods shares the common disadvantage of relying on homologous recombination of the target DNA into recipient chromosomal DNA; the necessary homologies may not always exist, and even where they do exist, the recombination events may be slow. In addition, each of the above percentages for successful DNA expression is decreased by a factor of approximately two if the only insertions counted are those that are successfully passed on to subsequent generations of cells or offspring.

Because the limiting factor for most transformations is typically the availability of embryos, it is desirable to optimize the chances of stable DNA insertion into the available embryos. An embryo, regardless of source, should be in the 2-4 cell stage of development to maximize the probability of a stable insertion that will be incorporated into the germ cells. Obtaining embryos at this stage requires careful timing; and a quick response time will often be necessary to ensure that the cells are manipulated while in the proper stage, before their development progresses too far.

Current procedures for the genetic transformation of higher organisms are not only time-consuming, but are also expensive in terms of person-hours used. For these and other reasons, there remains an unfilled need for a more efficient means of delivering and stably integrating exogenous DNA into the chromosomes of a higher organism.

Transposons

A transposon is a mobile genetic element capable of inserting at random into a DNA sequence. See generally Lewin, *Genes IV*, pp. 649-671 (1990). Most prior research on transposons has been conducted with bacteria; a limited number of studies have been conducted with transposons in eukaryotic cells. A wild-type transposon typically includes a gene encoding a transposase (an enzyme controlling transposition), flanked by two sequences called insertion sequences. It may also carry other genes, such as a gene for antibiotic resistance. The insertion sequences are generally inverted repeats of one another (exact or closely related). Wild-type transposons typically insert preferentially in certain regions or "hot spots" in the host genome.

Prior work with transposons has used transposons for creating mutations, e.g., disrupting existing genes; and in studying pathogenesis. In such applications, unmodified wild-type transposons have several disadvantages. These disadvantages include undesired homologous recombinations resulting from the relatively large size of the insertion sequences, and spontaneous transposition of a transposon to another location in the genome induced by the transposase. Spontaneous transposition is relatively rare, but it can cause the expression of a new phenotypic trait, making interpretation of results difficult.

"Mini-transposons" have recently been developed to attempt to circumvent some of the problems associated with wild-type transposons. Kleckner *et al.*, "Uses of Transposons with Emphasis on Tn10," pp. 139-180 in Miller (ed.), *Methods in Enzymology*, vol. 204 (1991). These mini-transposons have been modified from the wild-type transposons in three ways: (1) The transposase gene has been removed from its native site between the two insertion sequences, and instead placed upstream from the transposon. This rearrangement promotes a more stable insertion that should not move following insertion, because the transposase gene is lost upon insertion. (2) The insertion sequences have been shortened to about 70 base pairs in length, compared to sequences that are typically well over 100 base pairs in wild-type transposons. This shorter length greatly inhibits unwanted homologous recombinations. (3) The entire transposon "cassette" has been placed under the control of an inducible promoter, such as the *ptac* promoter. The *ptac* promoter is only read in the presence of isopropyl- β -D-thiogalactopyranoside (IPTG), allowing complete control over when the transposase is activated to cause transposition of the transposon. Furthermore, the transposase is often mutated so that it is less specific to "hot spots," sequences where the wild-type transposon preferentially inserts. This lowered specificity increases the rate of insertion into the genome.

Mini-transposons have previously been used in place of wild-type transposons to cause mutations by disrupting genes, and to study pathogenesis. It has not previously been suggested that a mini-transposon might be used as a vector for stably transforming an exogenous gene into a eukaryotic chromosome.

DISCLOSURE OF THE INVENTION

Novel means have been discovered for increasing the resistance of a mammalian (including human), vertebrate, or invertebrate host to diseases caused by intracellular bacteria, protozoa, and viruses. The infection treated may, for example, be equine infectious anemia, or infection by the human immunodeficiency virus. Novel means have also been found for treating tumors.

Augmentation of the host's defenses against infectious diseases or tumors is achieved by "arming" the host's cells with an exogenous gene. The host's own leukocytes, other cells involved in resistance to infection, or other cells are transformed with a gene conferring the ability to synthesize and secrete natural or synthetic lytic peptides, such as native cecropin B or synthetic lytic peptides such as SB-37, Shiva I through X, or Manitou. The expression of the genes is induced when needed to combat pathogens.

For example, the transfection of hematopoietic stem cells and embryonic cells will produce animals (or humans) with enhanced disease resistance; and transfection of leukocytes such as TIL (tumor infiltrating lymphocytes) cells, neutrophils, macrophages, or cytotoxic lymphocytes can be used in the treatment of tumors. Stable incorporation of the gene into an egg, a zygote, an early-stage embryo, or a totipotent embryonal stem cell will cause the gene to be carried in germ cells, and thus to be inherited by future generations. Such a transformed animal (e.g., a chicken or a catfish) would have economic significance

It has been discovered that genes coding for a cecropin or other native or synthetic lytic peptide, such as native cecropin B or Shiva 1, can be transferred and stably expressed in mammalian, other vertebrate, and other animal cells. The transformed cells have the ability to produce and secrete a broad spectrum chemotherapeutic agent that has a systemic effect on certain pathogens, particularly pathogens that might otherwise evade or overcome host defenses. The peptide's expression is preferably induced only in areas of infection, where it will most effectively augment the host's defense systems. Animal cells, including mammalian cells and fish cells, have been transfected with cecropin B, and will also be transfected with cecropin analogs. It has been observed that expression of the exogenous gene does not damage a healthy recipient cell.

Novel means have also been discovered for transforming a eukaryotic cell with a gene under the control of an exogenous promoter that is responsive to an inducer of an acute-phase peptide or protein. As an example, it was unexpected that the transfer of a gene coding for an invertebrate lytic peptide, or a synthetic homolog of such a peptide, can enhance the immune potential of a vertebrate animal. It is particularly unexpected that placing (or leaving) the gene under the control of a native invertebrate promoter such as the native *Hyalophora cecropia* promoter leads to appropriately induced expression in vertebrate cells. To our knowledge, the stable insertion and expression of a gene in a vertebrate cell under the control of an invertebrate promoter have never previously been accomplished. To our knowledge, the stable insertion and expression of a gene in a eukaryotic cell under the control of an exogenous promoter responsive to an inducer of an acute phase protein has never previously been accomplished.

A novel transposon-based vector has also been developed, a novel vector that enhances the integration of DNA into a host genome, particularly a eukaryotic genome. The novel vector has been used, for example, in the transformation of mammalian and fish cells with a gene coding for the lytic peptide cecropin B. The novel vector allows the rapid and efficient transformation of a eukaryotic genome. Its use does not require the high level of skill needed for microinjections. Nor does it rely on homologous recombination events for a successful transformation, as do the prior methods of microinjection, electroporation, and lipofection.

These and other advantages were achieved by constructing a novel modification of mini-transposons to adapt them to carry a gene of interest into a genome. Such an adaptation of mini-transposons has never previously been suggested.

Briefly, the novel vector for transforming an exogenous gene into a eukaryotic cell comprises: a gene encoding a transposase; two transposon insertion sequences; an exogenous gene, where the exogenous gene is located between the two insertion sequences; and a promoter that is adapted to cause the transcription of the transposase; where one of the insertion sequences is located between the transposase gene and the exogenous gene; and where the transposase is adapted to excise from the vector a fragment comprising the two insertion sequences and the portion of the vector between the two insertion sequences, and to insert the excised fragment into a chromosome of a eukaryotic cell.

This arrangement insures that the transposase gene is not incorporated into the target chromosome, insuring that the transformation will be stable. Descendants of a cell transformed with the vector will not have a copy of the transposase gene. Without a

transposase gene to encode a transposase, there will be nothing to promote excision of the exogenous gene from the genome.

The examples given below use a vector based on the Tn10 transposon, but analogous vectors constructed from other transposons will work in the present invention as well. Examples of transposons are known in the art, and include, for example, AC7, Tn5SEQ1, Tn916, Tn951, Tn1721, Tn2410, Tn1681, Tn1, Tn2, Tn3, Tn4, Tn5, Tn6, Tn9, Tn30, Tn101, Tn903, Tn501, Tn1000 ($\gamma\delta$), Tn1681, Tn2901, AC transposons, Mp transposons, Spm transposons, En transposons, Dotted transposons, Mu transposons, Ds transposons, dSpm transposons, and I transposons.

All transposons function in an essentially similar manner, so the transposon used to construct novel vectors in accordance with this invention is a matter of choice. The insertion sequences of a transposon will recognize their target sequences (typically 8-10 base pairs in length), regardless of the species in whose genome the target sequences are found; and they will insert into the target sequence the DNA lying between the flanking insertion sequences. Target sequences for a given transposon will occur in a genome at a statistically predictable frequency, such that it is statistically likely that any given eukaryotic genome will have multiple target sequences for the insertion sequences from any given transposon.

BEST MODE FOR CARRYING OUT THE INVENTION

General

Studies in our laboratory have shown that a variety of treatments make cells susceptible to at least some lytic peptides, in situations where corresponding untreated cells are resistant to the peptides. Resistant cells can be made susceptible by treatment with cytoskeletal inhibitors, cytochalasin D and colchicine, or by chilling the cells to 4°C for 15 minutes prior to exposure to peptide. Resistant cells treated with trypsin also became extremely susceptible to lysis by the lytic peptides. A common factor in each of these examples of induced susceptibility appears to be an altered plasma membrane and/or cytoskeleton. The alteration may interfere with the repair of damaged membrane by hampering endocytosis or exocytosis.

Further evidence of the selective susceptibility of macrophages to the lytic peptides was obtained in a series of experiments using mouse peritoneal macrophages and *Listeria monocytogenes*, an obligate intracellular, gram-positive bacterium. Normal, non-activated, resident peritoneal macrophages (R1); and activated macrophages derived from the peritoneal cavities of *Listeria*-immune mice inoculated intra-peritoneally 17 hours earlier with *Listeria* (L1) were exposed *in vitro* to *Listeria* and then treated with a lytic peptide.

Additionally, resident macrophages from normal non-*Listeria* immune mice were infected *in vitro* with *Listeria* (L2) and treated with the Shiva I peptide. The Shiva peptide had little effect on control resident macrophages (R1). Neither *Listeria* alone nor the peptide alone (without *Listeria* infection) resulted in significant cell death. However, *Listeria*-infected macrophages (L1 and L2) were killed by the peptide. Macrophages from *Listeria*-immune mice (L1) re-exposed to *Listeria* by intraperitoneal inoculation 17 hours earlier were killed when exposed to the peptide. The presence of intracellular *Listeria* was confirmed by microscopic examination of the groups of cells prior to treatment.

As described in greater detail below, we have developed a construct carrying the gene for the native cecropin peptide and the native cecropin promoter, a construct that can be inducibly expressed in animal cells, and that has been successfully expressed in mammalian cells and fish cells. This result is quite unexpected, particularly because a native insect promoter has been successfully used to regulate expression of a gene in mammalian cells. The construct was made so that the native gene, or synthetic genes for analogues of the native peptides, can be placed under the control of the native cecropin promoter.

The plasmid construct designated "pCEP" carries both the native cecropin promoter and the native cecropin gene. Electroporation of the pCEP construct into fetal donkey dermal cells ("FDD cells") resulted in the expression of antibacterial substances when those cells were co-cultured with viable *E. coli*. This antibacterial activity was not observed in control electroporated FDD cells.

Transformation of Fetal Donkey Dermal Cells

Fetal donkey dermal cells were chosen as a model system for cecropin expression. This cell line was chosen for several reasons. First, it was known from prior studies that these cells are resistant to lysis by lytic peptides. Second, this cell line had previously been used to study the antiviral activity of several lytic peptides against Equine Infectious Anemia (EIA) infection. The EIA-infected cells were lysed by the peptides, while uninfected cells were not. The cell line has been demonstrated to be refractory to damage from electroporation. Finally, these cells will act as hosts *in vitro* for *Listeria monocytogenes* and *Trypanosoma cruzi*, agents to be used to evaluate the anti-bacterial and anti-protozoal activity of cells expressing native or analog lytic peptides.

FDD cells were cultivated in Eagle's minimum essential medium (MEM), supplemented with Earle's salts, L-glutamine, nonessential amino acids, 5% fetal bovine serum (FBS), and the antibiotics penicillin (100 unit/ml) and streptomycin (100 µg/ml). Cells were split once a week until the desired number were obtained. Conditioned

medium that had been clarified from a freshly split culture of FDD cells after 24 hours of culture was used to maintain the cells after electroporation.

Prior to electroporation, FDD cells were rinsed with phosphate buffered saline (PBS), scraped from the flask, rinsed again with PBS, and counted. The concentration of cells was adjusted to 9×10^5 cells/100 μ L, and the cells were placed in a BioRad 0.4 cm (electrode gap width) electroporation cuvette. To this cuvette were also added 400 μ L of PBS and 1.4 μ g of linearized pCEP DNA. The cuvette and its contents were kept on ice until electroporation. The cells were electroporated at 2.0 KV and 1 μ F in the presence of 10 mM IPTG. Immediately after electroporation, 0.5 ml of conditioned medium was added to the cells, which were then incubated on ice for a 10 min recovery period. The cells were then transferred to flasks containing equal parts of conditioned medium and fresh medium, and were allowed to form a monolayer. Monolayered cells were trypsinized, subpassaged in 24-well plates, and allowed to form a monolayer. These cells were then subpassaged into 96-well plates. In the 96-well plates, the cells were grown without any antibiotics, and allowed to form a monolayer.

A two-step method was used to enrich for the population of cells expressing the antibacterial substance. It has been observed that antibacterial activity in cells expressing lytic peptides is associated with a loss of cellular sensitivity to trypsin. This trait allowed the selective removal of cells not expressing the antibacterial substance. Cultures were first exposed to *E. coli*, followed by exposure to trypsin. Cultures demonstrating antibacterial activity were scraped to remove the trypsin-resistant cells. These trypsin-resistant cells were then further diluted and subcultured. Those cells continued to divide to produce a monolayer culture of FDD cells resistant to bacterial colonization. Cultures of cells expressing antibacterial activity were demonstrated to contain the cecropin gene by Southern blotting. Electroporated cell monolayers unable to prevent bacterial colonization were presumed to be negative for expression of the cecropin gene.

Pathogen Challenges

When the monolayer was complete, a first challenge with pathogenic *E. coli* (isolated from a case of equine cystitis) was added at a concentration of 10 bacteria/well. This low concentration was chosen to attempt to stimulate cecropin production, without overwhelming the culture. In the second challenge, bacteria were added at a rate of 1000/well and incubated overnight. After incubation, the wells were examined for colonization of the bacteria in clumps on the surface of the FDD cell monolayer. Bacterial colonization ranged from wells with no bacterial colonies to wells overgrown with bacterial colonies. Wells in which there was no colonization or only slight colonization (about 15% of the total) were rinsed, and antibiotic medium was added back

to the wells. Cells were harvested and transferred to flasks to allow monolayer formation. It was observed that the FDD cultures that prevented colonization of bacteria also showed a loss of trypsin sensitivity. This same phenotypic trait had previously been observed in FDD cells following exposure to exogenous cecropin analogs.

5 The transformed FDD cells expressed the cecropin gene inductively, rather than constitutively. When the cells were split before exposure to bacteria, they were susceptible to treatment with trypsin; but after exposure to bacteria and subsequent cecropin production, the cells were resistant to trypsinization.

10 FDD cells positive and negative for antibacterial activity were expanded in 75 cm² flasks. Both groups of cells were challenged with 10³-10⁵ EIA viral particles and incubated at 37°C. Daily examination of the cells showed the negative control cells acting as normally-infected EIA cells. However, the FDD cells positive for antibacterial activity demonstrated an increased cytopathic effect, manifested approximately 3 days before that of the control cells. This increased cytopathic effect is believed to be due to cecropin
15 production by the FDD cells, a hypothesis that will be tested through a series of deletion mutations, as described below. These results demonstrate the usefulness of the present invention in treating virally-infected cells.

Electroporation of FDD cells has been repeated five times, and bacterial challenges have been performed on all five groups. Cells positive for antibacterial activity have been
20 detected in each of the groups. Cells from the earliest electroporations have been passaged numerous times; they have also been frozen and brought back to culture; all without any apparent loss in viability or phenotypic changes. The incorporation of DNA appeared to be stable: cecropin mRNA was detected in cells descended from the first electroporation that had subsequently been passaged four times.

25 *Confirmation of Transformation by Southern Blot*

Southern blot analysis was performed both on FDD cells that were positive for antibacterial activity, and on FDD cells that were negative for antibacterial activity. Electroporated cells not receiving DNA were used as negative controls in the Southern analysis. The chromosomal DNA was harvested from FDD cells using the protocol of
30 Ausubel *et al.*, Current Protocols in Molecular Biology, vols. 1 and 2 (1991) for tissue-culture cells, and that DNA was electrophoresed on a 0.8% agarose gel. The DNA was transferred from the gel to a positively charged nylon membrane (Zeta Probe GT; Bio-Rad Laboratories, Richmond, CA), where it was probed with the cecropin gene isolated from pMON 200. The probe was prepared, and the subsequent hybridization was performed,
35 using the non-isotopic Genius 1™ nonradioactive DNA labelling and detection kit (Boehringer Mannheim Corporation, Indianapolis, IN). The high stringency protocol was

performed according to the manufacturer's instructions. Positive hybridization results were observed only in the electroporated cells receiving pCEP DNA, and in the pCEP vector used as a positive control. No hybridization was seen in the electroporated cells that did not receive pCEP DNA.

Confirmation of Transformation by PCR

FDD cells positive for cecropin were rinsed with PBS and fed with MEM that contained antibiotics as described above. The positive clones were passaged three times to try to insure that no cecropin or associated mRNA remained in the cells. After the third passage formed a monolayer, the cells were split into two groups. One group was challenged with bacteria, and the second group received a PBS treatment without bacteria as described above. After a 24-hour incubation, both groups were harvested, and RNA was harvested by the method of Ausubel *et al.*, Current Protocols in Molecular Biology, vols. 1 and 2 (1991). Briefly, 3.5 ml of 4 M guanidinium thiocyanate solution was added per each 10^8 cells, both to lyse the cells and to inactivate any RNase present. The resulting lysate was suspended in 5.7 M cesium chloride, and centrifuged at $150,000 \times g$ for 16 hours to separate RNA from DNA. The RNA pellet was resuspended in TES (10 mM TrisHCl, pH 7.4; 5 mM EDTA; 1% sodium dodecyl sulfate (SDS)), 3 M sodium acetate, and 100% ethanol, and then placed on dry ice/ethanol for 30 min to precipitate the RNA. The pelleted RNA was resuspended in 200 μ L of sterile, distilled water, and quantitated by measuring absorbance at 260 nm and 280 nm.

The poly(A)-RNA (i.e., mRNA) was then separated from the tRNA and rRNA as follows. Total RNA was denatured by heating to 70°C for 10 minutes to expose any poly(A)+ sites, and to disrupt secondary structures. The RNA mixture was passed through an oligo(dT) column to bind the poly(A)+ sites. The column was then washed twice to remove rRNA and tRNA, and then 2 mM EDTA/0.1% SDS was used to elute the mRNA. The mRNA was precipitated with ethanol and sodium acetate, and resuspended in TE (10 mM TrisHCl, pH 8.0, and 1 mM EDTA).

The mRNA was then used for PCR amplification using the semi-quantitative protocol of Dallman *et al.*, "Semi-quantitative PCR for the analysis of gene expression," in Rickwood *et al.* (eds.), *PCR: A Practical Approach* (1991). Briefly, synthesis of cDNA from the mRNA was performed with reverse transcriptase from Moloney murine leukemia virus (Gibco-BRL). Using primers to the prececropin gene sequence, the cDNA was then amplified via PCR: cycle at 94°C, 1 min (denaturing); at 55°C, 2 min (annealing); and at 72°C 1 min (extension). After 10-20 cycles, 15 μ L samples can be taken at the end of every 5th cycle, and stored in 96-well microtiter plates. Quantitation of the unknown cDNA was achieved by having internal oligonucleotide standards that

were titrated against the cDNA. The concentration of the standard at which the amount of amplification product was equal to the amount of amplification product from the target approximated the starting concentration of the experimental DNA (to within, say, an order of magnitude).

One group of transformed FDD cells was challenged with bacteria for 6 hours, after which the cells were harvested for mRNA isolation. Purified mRNA was reverse-transcribed to cDNA using Moloney Murine virus reverse transcriptase. Following the procedure described above, the cDNA was added to a PCR-amplification mixture with primers to pre-cecropin B, and was cycled 30 times in the thermocycler. FDD cells without vector DNA were used as negative controls. The mRNA from one group of cells showing antibacterial activity had a 180 bp fragment that corresponded to the size that was expected to be amplified, based on the design of the primers. This band was not present in the control cell mRNA, nor in the non-challenged cecropin-transformed FDD cells, showing that the cecropin was not constitutively produced.

Acute Phase Response Mechanisms

Our results demonstrate the ability of FDD cells to recognize the native cecropin promoter, and to express the cecropin B peptide in response to exposure to pathogenic *E. coli*. Replication of the cells was not affected. More generally, we expect that a wide variety of animal cells will express genes placed under the control of exogenous promoters responsive to inducers of acute-phase proteins or peptides, due to the high degree of homology many such proteins and peptides have maintained across widely-separated taxa.

Without wishing to be bound by this theory, the fact that the insect promoter was appropriately induced in a mammalian cell suggests that there is substantial homology between the acute phase response (APR) mechanisms of insect cells and mammalian cells - sufficient homology that the mammalian cells recognize the insect promoter and express the gene controlled by that promoter.

Various APR's from various species that have been identified to date share certain similarities at the genetic level, similarities that may be related to their transcription. Known IL-1 sequences, tumor necrosis factor (TNF), human lymphotoxin (LT), human and mouse granulocyte-macrophage colony stimulating factors (GM-CSF), and fibronectin sequences have a common 8 base sequence -- TTATTTAT -- in the region of the gene that is transcribed into an untranslated portion of the 3' end of the mRNA. Although the function of this sequence is not known, it is believed to influence the termination codon in some fashion; in homologous molecules in different organisms, e.g., human and mouse IL-1, the distance to the termination codon is conserved. Alternatively, it may serve as a possible target for endoribonucleases involved in the rapid removal of mRNA when

inflammation ceases. Caput *et al.*, "Identification of a common nucleotide sequence in the 3'-untranslated region of mRNA molecules specifying inflammatory mediators," Proc. Natl. Acad. Sci. USA, vol. 83, pp. 1670-1674 (1986); Toniatti *et al.*, "Regulation of the Human C-reactive Protein Gene, a major marker of Inflammation and Cancer," Mol. Biol. Med., vol. 7, pp. 199-212 (1990). This conserved octamer has recently been identified in the gene for the LPS-binding protein of the American cockroach, *Periplaneta americana*. Jomori *et al.*, "Molecular Cloning of cDNA for Lipopolysaccharide binding Protein from the Hemolymph of the American Cockroach, *Periplaneta americana*," J. Biol. Chem. vol. 266, pp. 13318-13323 (1991).

Another common APR sequence in the portion of the gene corresponding to the 3' untranslated region of the mRNA's appears to be the sequence ATTTA, a sequence that is believed to be responsible for unstable mRNA. Jomori *et al.*, "Molecular Cloning of cDNA for Lipopolysaccharide binding Protein from the Hemolymph of the American Cockroach, *Periplaneta americana*," J. Biol. Chem. vol. 266, pp. 13318-13323 (1991). Also identified as conserved regions in many APR's are gene sequences corresponding to non-translated sequences in the 5' end of the mRNA's. One such sequence is TGGRAA, which has been reported for α 2-macroglobulin, α 1-acid glycoprotein, γ -fibrinogen, haptoglobin and human C-reactive protein (CRP). Dente *et al.*, "Structure of the Human α 1-acid glycoprotein gene: sequence homology with other human acute phase protein genes," Nucl. Acids Res., vol. 13, pp. 3941-3952 (1985); Fey *et al.*, "Regulation of rat liver Acute-Phase genes by Interleukin-6 and production of hepatocyte stimulating factors by rat hepatoma cells," Ann. N.Y. Acad. Sci. vol. 557, pp. 317-331 (1989); Toniatti *et al.*, "Regulation of the Human C-reactive Protein Gene, a major marker of Inflammation and Cancer," Mol. Biol. Med. vol. 7, pp. 199-212 (1990). Other conserved 5' transcription factors include the CCAAT/enhancer binding protein (C/EBP) and the CCATT box and enhancer core sequences which are common in a wide range of APR associated gene sequences. Kaling *et al.*, "Liver-Specific Gene Expression: A-Activator-Binding Site, a Promoter Module Present in Vitellogen and Acute-Phase Genes," Mol. Cell. Biol., vol. 11, pp. 93-101 (1991).

Conserved APR sequences for other molecules were compared to Xanthopoulos' published sequence for the native cecropin. Xanthopoulos *et al.*, "The structure of the gene for cecropin B, an antibacterial immune protein from *Hyalophora cecropia*," Eur. J. Biochem., vol. 172, pp. 371-376 (1988). Though not described by Xanthopoulos *et al.*, several sequences were found corresponding to conserved sequences for other APR's. The first conserved sequence found was in the 3' untranslated region -- TTATTTAT -- found at positions 814 - 821 and at 972-979, and found again at position 848 - 855 with

only one base substitution. The conserved sequence ATTTA corresponding to unstable mRNA is found in the 3' untranslated region a total of six times. In the 5' untranslated region of the cecropin gene, the sequence TGGRAA is found twice with one base substitution in each sequence, but whether this sequence is functional remains to be determined. A conserved sequence corresponding to the CCATT box and to the enhancer core is found two times in the 5' untranslated region of the gene. With the benefit of hindsight, these homologies make plausible our discovery that the native cecropin B promoter and peptide gene respond to at least some vertebrate APR inducers to elicit expression in vertebrate cells.

It is believed (without wishing to be bound by this theory) that an as-yet-unidentified cytokine is responsible for eliciting both cecropin production and secretion. Unpublished data from our laboratory suggests that cecropin is produced in the moth in response to the presence of a cytokine. Injection of the pupal stage of the giant silkworm moth with either *E. coli* or *E. coli* LPS (lipopolysaccharide or endotoxin), or with human recombinant interleukin-1 resulted in hypertrophic changes in the fat body of the pupae, and in elevated levels of cecropin in the hemolymph. Without wishing to be bound by this theory, it is believed that a cytokine or similar inducing agent is responsible for inducing expression of the gene in vertebrate cells as well when the native moth promoter is used to control the gene.

The Cecropin Promoter

The function of the cecropin promoter will be studied by inserting the neo-CAT gene (chloramphenicol acetyl transferase, a reporter gene) under its control in place of the lytic peptide gene. Transformed cells will be screened by assaying for CAT enzyme activity. The neo-CAT gene was chosen because it will allow pre-selection of FDD cells containing the CAT gene by selecting in the presence of neomycin. Once a transformed cell line with the CAT gene is established, cecropin promoter activity will be studied by altering the promoter with point and/or deletion mutations in the conserved promoter binding sequences, and assaying for altered CAT expression. The neo-CAT gene will be digested from the vector pMAMneo-CAT (Clontech, Palo Alto, CA) and inserted into the Sal I site of pCEP to replace the cecropin peptide gene. The conserved 5' APR signals in the cecropin promoter will be altered by oligo-directed mutagenesis as described by Zoler *et al.*, Methods Enzymol., vol. 100, pp. 468-500 (1983). Briefly, ssDNA from construct 5'Δ-94 will be used as a template for the *in vitro* synthesis of the complementary strand. The mutations are designed to insert into a Bal I restriction site. A set of synthetic oligonucleotides carrying the base substitution(s) are used as primers for the second strand. The mutant cecropin promoter is then cloned into the Bal I site of pCEP linked to

the CAT gene. Using this approach, substitutions will be created in the conserved CCATT, TTGGACA, and TTGGAAC sequences of the promoter.

Electroporation will be performed as described earlier, and enzyme production will be screened by SDS-PAGE and Western blot analysis with rabbit anti-CAT. The intact cecropin gene (including the unaltered promoter) will be run simultaneously in all assays for comparison. Identification of the antibacterial substance as cecropin B, and its localization within FDD cells and on their surface, will be accomplished using rabbit anti-cecropin B polyclonal antibodies with an indirect fluorescent antibody procedure.

The intact cecropin B gene and the neo-CAT gene in pCEP will be run concurrently in the following experiments to probe the means of expression. Cecropin- and neo-CAT-producing FDD cells will be prepared as described above using the bacterial, LPS, or IL-1 challenge method. Cells positive for cecropin and neo-CAT will be rinsed with PBS and cultured in MEM containing antibiotics. The positive clones will be passaged three times to insure that no cecropin, CAT enzyme, or RNA's associated with their production are left in the cells. After each third passage forms a monolayer, the cells will be split into two groups; one group will be challenged with bacteria in PBS, and the second group will receive a PBS treatment without bacteria. After a 24-hour incubation, the RNA from both groups will be harvested as described by the method of Ausubel *et al.*, Current Protocols in Molecular Biology, vols. 1 and 2 (1991), as described above.

The mRNA is then used for PCR amplification using the semi-quantitative protocol previously described, using primers to the pre-cecropin and CAT gene sequences. If, as expected, cecropin and CAT expression is induced, then only cells exposed to the bacteria or cytokine will yield positive results for the mRNA. If, however, either protein is constitutively produced, then both the negative controls and the "induced" cells will demonstrate a positive response for mRNA; and if this is the case the quantitative portion of this experiment will determine whether the levels increase in response to the inducing agent.

To understand how the cell is induced to produce cecropin, responses to various agents will be measured. The same experiments will be conducted on cells containing vectors in which the cecropin promoter has been altered. In the unlikely event that the cecropin promoter is determined not to be the promoter responsible for cecropin production, then the primers made to the cecropin and/or CAT genes will be used to sequence into the FDD genome to identify and characterize the active promoter. Purified LPS (Sigma Chemical) will be added to cells negative for cecropin production; the supernatant will be collected; and the LPS will be removed with neutralizing antibody (anti-LPS). Four groups of cells will then be exposed to different inducing agents: (1)

LPS-induced-cell supernatant treated with anti-LPS and polymyxin B to remove LPS; (2) IL-1 added to the FDD cells; (3) FDD cells induced with whole bacteria; and (4) FDD cells receiving a PBS placebo as a control. The supernatant will be collected from each group and split into two groups: half of the supernatant will be used to determine cecropin activity on *E. coli* or CAT activity, and the other half will be quantitated for the amount of cecropin or CAT production using a polyclonal anti-cecropin or anti-CAT. Some of the supernatant from the LPS-induced group and the IL-1-induced group will be treated with anti-IL-1 and used to challenge cecropin-producing cells. Cecropin or CAT production will be measured using the bacterial assay and the polyclonal antibody to cecropin or CAT.

If LPS alone is responsible for inducing cecropin expression, then only the LPS and bacteria-induced FDD cells should show a positive cecropin or CAT response. If IL-1 is responsible for induction, then all of the cells except the control should show a positive response, and this response should be neutralized with anti-IL-1, which would then yield a negative result for cecropin or CAT when the supernatant is used to induce FDD cells. Other cytokines (e.g., IL-6, TNF (Biogen), IL-2 (Bioferon), and purified γ -IFN (Bioferon)) will be assayed in a similar fashion.

Other Promoters

Promoters other than the native cecropin promoter are suitable for use in the present invention. Such promoters include other native promoters for native lytic peptides, and other native promoters that are responsive to APR inducers. The identification and isolation of such promoters is within the ability of one of ordinary skill in the art, given the teachings of the present specification, including particularly the following discussion.

Where such promoters are not already identified in the literature, they may be identified as follows. With a lytic peptide, the DNA upstream from the coding region of the gene may be sequenced, and the portion comprising the promoter identified through standard means, by searching for conserved sequences that are typical of promoters.

APR inducers, such as IL-1, IL-2, IL-6, C-reactive protein, LPS binding protein, lymphotoxin, granulocyte-macrophage colony stimulating factors, fibronectin, interferons, and tumor necrosis factor, induce other genes as well. Genes induced by an APR inducer may be identified through means known in the art, including isolation of the proteins thus translated, or identification of induced mRNA's through subtractive hybridization. Where the protein is isolated, through means known in the art: it may be partially sequenced; a probe for a genomic library or (preferably) for an appropriate cDNA library may be prepared; and the cloned gene may then be sequenced, including its promoter region.

Construction of Plasmid pCEP, and Transfection of E. coli with pCEP

The plasmid pCEP is a pBR322 derivative that carries the gene for ampicillin resistance and the ColE1 origin of replication, but in which a segment from base pair 105 to base pair 2345 has been deleted to streamline the plasmid, to allow incorporation of the native cecropin promoter and gene, or other gene of interest. The cecropin gene segment is the 5.9 Kb fragment isolated from the vector pMON 200 by a restriction digest with EcoR I and Xho I. Both ends were filled to create a blunt-ended fragment, as were the ends of the pBR322 plasmid.

The native cecropin gene was ligated into the modified pBR322 vector to give a construct of 9.3 Kb, using a modification of Ausubel *et al.*, Current Protocols in Molecular Biology, vols. 1 and 2 (1991); and Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, 2nd ed. (1989). The modification, discussed further below, increases the chance of blunt-end fragment insertion into the plasmid. The resulting recombinant plasmid, designated pCEP, was then electroporated into *Escherichia coli* NM554 (Stratagene, La Jolla, CA) using a BioRad Gene Pulser™ electroporator, under conditions described by the manufacturer for *E. coli*. Electroporated bacteria were plated onto Brain-Heart Infusion (BHI) agar containing 50 µL/ml of ampicillin, and incubated at 37°C overnight. Because the *ptac* promoter is not read unless induced, the potentially lethal lytic peptide gene may be maintained in *E. coli* without the danger that the peptide will kill the *E. coli*.

It is preferred that the promoter controlling the transcription of the transposase be inducible, so that the transposase gene is not transcribed until an inducing stimulus (e.g., IPTG for the *ptac* promoter) is supplied. However, it would also be possible to use a constitutive promoter for the transposase, particularly a promoter that is inactive in *E. coli* or other prokaryotic host, but that is expressed constitutively in a eukaryotic cell. The vector would be lost or diluted following a number of cell divisions, so that continuing transpositions of the inserted segment should not occur.

Colonies growing on the BHI/ampicillin plates were subcultured in BHI/ampicillin broth for plasmid screening (Qiagen, Chatsworth, CA) and freezing at -70°C. Plasmid preparations of the isolates were examined by agarose gel electrophoresis. A 1% gel was electrophoresed for 4 hours at 4 V/cm, stained with 0.4 µg/ml ethidium bromide for 10 min., and destained in distilled water for 30 min. A supercoiled plasmid DNA ladder (Sigma Chemical) was used as a DNA size reference; bands corresponding to ~9.3 Kb were removed from the agarose and purified using the Eluquick™ DNA Purification Kit protocol. Purified plasmid DNA was then electroporated back into competent *E. coli* NM554 and selected on BHI/ampicillin plates. Because *E. coli* NM554 is a plasmid-less

strain, this additional purification-electroporation step insured that there was only one plasmid type per cell by eliminating pBR322-pBR322 self-ligated dimers.

Confirmation of the pCEP plasmid was obtained by growing *E. coli* NM554 on BHI/ampicillin plates; making plasmid preparations; making restriction digests with BamH I to yield two fragments of 2.3 and 7.0 Kb; and Southern blotting of the restriction digest under very stringent conditions using the cecropin gene isolated from pMON 200 as a probe.

A sample of this transformed *E. coli* strain NM554 containing plasmid pCEP with the cecropin gene was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852 on June 30, 1993, and was assigned ATCC Accession No. 69342. This deposit was made pursuant to a contract between ATCC and the assignee of this patent application, Board of Supervisors of Louisiana State University and Agricultural and Mechanical College. The contract with ATCC provides for the permanent and unrestricted availability of the progeny of this *E. coli* strain to the public on the issuance of the U. S. patent describing and identifying the deposit or the publication or the laying open to the public of any U.S. or foreign patent application, whichever comes first, and for availability of the progeny of this *E. coli* strain to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto under pertinent statutes and regulations. The assignee of the present application has agreed that if the *E. coli* strain on deposit should die or be lost or destroyed when cultivated under suitable conditions, it will be promptly replaced on notification with a viable culture of the same *E. coli* strain. As the term is used in the claims below, it should be understood that the "pCEP" plasmid encompasses not only the specific plasmid included in this ATCC deposit, but also any plasmid that is substantially identical to that plasmid, with the specific exception that the gene placed under the control of the cecropin promoter may vary; it is intended that the term "pCEP" plasmid should encompass any such plasmid, regardless of the specific DNA inserted.

After confirmation of the pCEP plasmid was completed, a large scale plasmid isolation was performed. A 250 ml culture of the transformed *E. coli* was grown in BHI/ampicillin broth on a rotary shaker at 37°C until an absorbance at 600 nm of $A_{600} = 0.4$ was obtained, at which time chloramphenicol was added to give a final concentration of 180 $\mu\text{g/ml}$ before amplifying the plasmid. Shaking was continued overnight. After 24 hours, the pCEP DNA was harvested using the Qiagen plasmid isolation protocol (Qiagen, Chatsworth, CA) and column to obtain pure, high quantity (0.7 $\mu\text{g}/\mu\text{L}$) plasmid DNA. A restriction digest using Pst I was conducted on 20 μg of the pCEP DNA to linearize the

DNA prior to electroporation into a mammalian cell line as described above. The enzyme Pst I was chosen because it linearizes the plasmid without cutting the cecropin gene.

The pCEP plasmid itself was constructed as follows. Plasmid pNK2859 containing Tn10 derivative 103 (obtained from Dr. Nancy Kleckner, Department of Biochemistry and Molecular Biology, Harvard University; see Kleckner *et al.*, "Uses of Transposons with Emphasis on Tn10," pp. 139-180 in Miller (ed.), *Methods in Enzymology*, vol. 204 (1991)) was digested with the enzyme BamH I. This digestion had the effect of removing the kanamycin antibiotic resistance marker from the transposon, but leaving the insertion sequences flanking the kanamycin resistance gene intact. The digest resulted in two bands approximately 3.2 Kb and 1.6 Kb in size. A double digestion was performed on the pMON 200 vector with the enzymes EcoR I and Xho I, yielding fragments of approximately 6 Kb and 9.7 Kb. These two enzymes remove the native cecropin B gene intact from the pMON 200 vector in the 6 Kb fragment. The resulting fragments from the two digests were separated by agarose gel electrophoresis on a 1% gel run at 40 V for four hours. The 3.2 Kb fragment from the transposon vector, and the 6 Kb fragment from the pMON 200 vector were excised from the gels, and each was separated from the agarose using the Eluquick™ DNA Purification Kit (Schleicher and Schuell, Keene, NH). This method minimized DNA shearing, and allowed efficient recovery of the desired fragments.

The purified DNA fragments did not have complimentary ends, so a blunt-end ligation protocol was designed, based on modifications of Ausubel *et al.*, *Current Protocols in Molecular Biology*, vols. 1 and 2 (1991); and Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, 2nd ed. (1989). Briefly, blunt end fragments were created using Klenow fragment and dNTP's (final concentration of 0.5 mM) at an incubation temperature of 30°C for 15 min, followed by deactivation at 75°C for 10 min. Both the cecropin fragment and the transposon vector fragment were extracted in phenol:chloroform; precipitated in isopropanol; and resuspended in 10 µL of TE buffer (10 mM Tris, 5 mM EDTA, pH 8.0). The cecropin fragment was then ligated onto the transposon vector fragment using T4 DNA ligase and 40% PEG (polyethylene glycol) according to Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, 2nd ed. (1989). PEG was used to enhance the ligation of blunt-ended fragments, and to minimize the formation of concatemers of the same DNA.

The ligation mixture was incubated overnight at 20°C. The following day DNA was extracted in phenol:chloroform; precipitated in isopropanol; and resuspended in 10 µL of sterile, distilled H₂O. *E. coli* NM554 (Stratagene, La Jolla, CA) was made competent for electroporation using the protocol of BioRad, Inc (BioRad Laboratories, Hercules,

CA). Electroporation was conducted at 1.65 KV, 200 Ω , and 25 μ F using 10^{10} cells of competent *E. coli*. Two electroporations were performed: (1) 10^{10} cells with 5 μ L of the ligated cecropin/vector DNA, and (2) a negative control using 10^{10} cells with 5 μ L of sterile, distilled H₂O. Each electroporation mixture was immediately placed in 1 ml of BHI (Brain Heart Infusion, Difco Inc.) broth at 37°C for one hour to recover, and to begin expression of the ampicillin resistance marker. After the one-hour recovery period, 200 μ L of each mixture was applied to an agar plate using the spread plate technique to cover the entire plate. *E. coli* receiving the ligated pCEP plasmid were plated onto BHI agar plates supplemented with 100 μ g/ml of ampicillin, while the *E. coli* receiving the distilled H₂O were plated both on BHI agar plates with ampicillin, and on BHI agar plates without ampicillin as controls. All plates were incubated overnight at 37°C. All colonies growing on the BHI/ampicillin agar plates were then grown in BHI/ampicillin broth, and frozen at -70°C.

Each colony was also screened for the presence of a plasmid of approximately 9.391 Kb: 5.99 Kb from the cecropin gene segment, plus 3.401 Kb from the vector DNA. When suitable candidates were identified, the potential pCEP-bearing *E. coli* were propagated in a 100 ml culture of BHI/ampicillin broth grown to an absorbance of $A_{600} = 0.4$, at which time chloramphenicol was added to a final concentration of 170 μ g/ml. The cultures were allowed to continue shaking overnight at 37°C for plasmid replication. The pCEP DNA was then harvested using a modified version of the Qiagen (Qiagen, Chatsworth, CA) protocol. Insertion of the cecropin B gene was confirmed using Southern blot analysis, with labelled cecropin gene as a probe. The nonisotopic Genius 1™ nonradioactive DNA labelling and detection Kit (Boehringer Mannheim) was used to perform the Southern analysis, and each step was conducted under stringent hybridization conditions. Isolates positive for the cecropin insert were then tested for the production of cecropin B as described above.

Other constructs will also be made. The first of these constructs is a streamlined native cecropin promoter-cecropin B peptide vector. The gene sequence obtained from the pMON 200 vector is rather cumbersome to work with, in that it is 6 Kb long. Using the published sequence and restriction map of cecropin B (Xanthopoulos *et al.*, "The structure of the gene for cecropin B, an antibacterial immune protein from *Hyalophora cecropia*," Eur. J. Biochem., vol. 172, pp. 371-376 (1988)) a construct will be made that includes the entire gene, and that is about 3.1 Kb, about half the size of the 6 Kb sequence. DNA from the pMON 200 vector will be partially digested with EcoR I and BamH I. The fragments will be separated on an agarose gel, and purified with the Eluquick™ DNA Purification Kit protocol described above. These fragments will then be cloned into a

pBR322-derived plasmid with polylinkers with either EcoR I- or BamH I-complementary ends to the digested fragment to insure proper orientation in the construct.

Streamlining the cecropin insert will allow easier removal of the gene encoding the native peptide, to facilitate replacement with a gene encoding a synthetic peptide such as Shiva I. If needed, another promoter can be substituted for the native cecropin promoter through means known in the art. At this time, however, the native cecropin promoter is preferred.

Creating a smaller delivery vehicle for the cecropin gene will also aid in sequencing the gene in a host cell using nucleic acid amplification techniques such as the polymerase chain reaction ("PCR"). Primers can be synthesized that will amplify internal segments of the cecropin gene, or segments extending into the host genome, to determine both the orientation and location of the gene in the host chromosome. A smaller fragment will enable sequencing with less time and expense.

Another Example -- Disease-resistant catfish

Catfish farming has rapidly become a major agricultural industry in the southeastern United States, particularly in Alabama, Louisiana, Mississippi, Arkansas, and South Carolina. A major factor limiting the economic success and future growth of the catfish industry is disease. Bacterial disease is the primary cause of mortality in commercially-reared channel catfish (*Ictalurus punctatus*), accounting for 57.5% of 9575 total cases examined from 1987 to 1989 by diagnostic labs in Alabama, Mississippi, and Louisiana. Infectious diseases cause an estimated 10% annual mortality in an industry where each 1% loss to disease translates to about a 5.5% loss of profits. The catfish industry could be greatly enhanced if this 55% loss of profits could be reduced.

Vaccination is one possible mechanism for reducing disease caused by bacterial or other pathogens. Of the major bacterial diseases associated with commercially-raised channel catfish, *Edwardsiella ictaluri* is the leading cause of mortality, followed by *Cytophaga columnaris* and *Aeromonas* sp. Some progress has been made in live attenuated vaccine construction for *E. ictaluri* and *A. hydrophila*. Though a marketable vaccine may be available in the near future for *E. ictaluri*, a vaccine for *A. hydrophila* is many years away, and a live attenuated vaccine for *C. columnaris* may never be feasible due to the difficulty in obtaining viable, stable mutants capable of eliciting an immune response. There remains an unfilled need for improved, inexpensive methods of combatting bacterial and other disease in catfish.

An alternative to creating a vaccine for each pathogen plaguing the catfish industry is to create a transgenic strain of catfish having lymphocytes capable of destroying invading organisms before the disease process is established. Toward this end, we have

transformed catfish zygotes with the cecropin B gene, using the novel transposon-based vector.

Channel catfish broodstock were obtained from the Aquaculture unit of Louisiana State University. Ripe females were dried of excess water and hand stripped of eggs. Before fertilization, eggs were electroporated with plasmid pCEP as described below. Fertilization was accomplished by mixing sperm into the electroporated eggs, which were allowed to water-harden for approximately 15 minutes. Once water-hardened, zygotes were treated with a 1.5% sodium sulfite solution to remove the gelatinous mass, and to inhibit possible fungal colonization. The 1.5% sodium sulfite solution was first adjusted to the pH of the water containing the zygotes. The sodium sulfite solution was then decanted from the zygotes, followed by a water rinse to remove excess sodium sulfite. Zygotes were then placed in hatching jars in a recirculating raceway with aeration, where they were maintained until hatching.

Electroporation was conducted with the BioRad Gene Pulser™ electroporator (Richmond, CA). Up to 1000 eggs were rinsed in Hank's buffer and placed in a specially designed cuvette with 3 ml of DNA solution. Eggs were pulsed 3 times using a 0.25 mC capacitor. Pulse field strength was 125 V/cm, and the time constant was 7-10 msec, with a pulse interval of 1 sec. Control electroporation was conducted with Hank's buffer alone. The DNA solution was removed, the eggs were rinsed in distilled H₂O, fertilized as described above, and placed in hatching jars for development.

Surviving fry were acclimated to 38-L aquaria on recirculating systems. Fry were fed a 50% protein diet two times a day for a total of 3% of their body weight. Fish were allowed to grow for 6 weeks before bacterial challenge.

Challenges were conducted with a known virulent strain of *E. ictaluri*. Fish that were potentially transgenic, the group of control electroporated fish, and a group of non-electroporated control fish were exposed to the pathogen by means of an immersion bath. Briefly, *E. ictaluri* was grown to completion in Brain-Heart Infusion broth (BHI; DifCo, Inc.), to about 10^{11} cells/ml. A 200 ml aliquot of the cells was added to each 38 liter tank to yield an inoculum of 5×10^8 cells/ml. Six-week-old fry were allowed to swim in the inoculum for 2 hours with aeration but no filtration. Tanks were examined 3 times a day for two weeks to count and remove dead fish. Dead specimens from each group were kept for tissue sampling.

The fish raised from the pCEP-electroporated cells had a substantially higher survival rate following the bacterial challenge than did either control group, suggesting that the transformation was successful, and that the cecropin B gene was appropriately expressed in the fish to combat bacterial infection.

Tissues were randomly sampled for subsequent DNA isolation and PCR amplification to screen for cecropin B insertion. Fish surviving the bacterial challenge were assumed to be transgenic. These fish were tagged, and a tissue sample was removed from the tail fin for DNA isolation and PCR analysis. PCR analysis revealed the presence of the cecropin B gene.

From the eggs that survived the electroporation to yield viable fry, 38 fry were randomly selected to be raised in a laboratory aquarium. Two fry died in transport, leaving a population of 36 on which to conduct tests. All 36 were subjected to the bacterial challenge, with 23 surviving the challenge.

Each of the 23 surviving fish was assayed for the presence of the cecropin B gene via the polymerase chain reaction (PCR). In addition to these 23 potentially transgenic fish, also assayed were four non-transgenic control catfish, and one spiked control sample containing non-transgenic catfish DNA mixed with the pCEP cecropin B vector. PCR primers 22 and 24 bases long for the cecropin B gene sequence were designed using PC Gene™ software (IntelliGenetics, Inc., Mountainview, CA). Using standard procedures, genomic DNA was isolated from blood drawn from anesthetized catfish. The PCR reactions were conducted with standard reaction mixtures: 100µl reaction mixture containing MgSO₄, DMSO, 10x buffer, genomic DNA, forward and reverse primers, dNTP's, distilled H₂O, and AmpliTaq™ DNA polymerase (Perkin-Elmer Cetus Corp.) PCR cycle parameters were as follows: denaturing at 95°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 60 seconds. The reactions were allowed to continue for 60 cycles in a Perkin-Elmer DNA thermocycler. After the PCR was complete, the samples were electrophoresed on a 2% agarose gel (50 ml volume) containing 1.5 µl of ethidium bromide solution (10 mg/ml stock solution), for 2 hours at 60 volts using a BioRad Model 200/2.0™ power supply.

The spiked control sample had a strong band at the approximate expected molecular weight (estimated by comparison to known molecular weight markers). Sixteen of the twenty-three experimental fish also showed a band at the same position. The negative control samples were all negative for this band; and in fact, were negative for any bands except for primer-dimer formation at the bottom of the gel.

Thus the genetic transformation with the cecropin B gene was successful in 16 of 23, or 70% of the fish surviving the bacterial challenge. The transformation was successful in at least 16 of 36, or 44% of the original pre-challenge population.

To date we have attempted genetic transformation with the pCEP vector in six experiments with fetal donkey dermal cells, as described generally above (although the experimental details were not identical in each of the six transformations). To date we

have attempted genetic transformation with the pCEP vector in one experiment with channel catfish. Each of these seven attempts has successfully produced cells transformed with the cecropin B gene. To date, we have experienced no unsuccessful attempts; that is, no attempted transformation of eukaryotic cells with the pCEP vector in our laboratory has failed to produce at least some cells successfully transformed with the cecropin B gene.

Experiments to detect cecropin B mRNA from blood drawn from the same catfish will be performed using standard techniques known in the art (see, e.g., the procedures described above to detect mRNA from FDD cells). These experiments are expected to demonstrate the expression of the cecropin B gene in the transformed catfish.

Miscellaneous

The complete disclosures of all references cited in this specification are hereby incorporated by reference, as are the complete disclosures of the two priority applications: U.S. patent applications S.N. 08/085,746 and 08/084,879, both of which were filed 30 June 1993.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>23</u> , line <u>11</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution <u>American Type Culture Collection</u>	
Address of depositary institution (including postal code and country) <u>12301 Parklawn Drive</u> <u>Rockville, MD 20852</u> <u>United States of America</u>	
Date of deposit <u>30 June 1993</u>	Accession Number <u>69342</u>
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
<u>E. coli 9063. Growth in BHI/ampicillin (100 µg/ml). Storage in BHI/Glycerol.</u> <u>Ampicillin resistant, Gram negative rod.</u>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

<div style="text-align: center; border-bottom: 1px solid black; margin-bottom: 5px;">For receiving Office use only</div> <div style="margin-bottom: 10px;"><input checked="" type="checkbox"/> This sheet was received with the international application</div> <div>Authorized officer <u>DORIS L. BROOK</u> <i>DLB</i> INTERNATIONAL DIVISION</div>	<div style="text-align: center; border-bottom: 1px solid black; margin-bottom: 5px;">For International Bureau use only</div> <div style="margin-bottom: 10px;"><input type="checkbox"/> This sheet was received by the International Bureau on:</div> <div>Authorized officer</div>
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What is claimed:

- 1 **1.** A transformed animal cell comprising a gene capable of expressing an
2 exogenous lytic peptide or a synthetic lytic peptide.
- 1 **2.** A cell as recited in Claim 1, wherein said cell comprises an embryonal stem
2 cell.
- 1 **3.** A cell as recited in Claim 1, wherein said cell comprises a mammalian cell.
- 1 **4.** A cell as recited in Claim 3, wherein said lytic peptide is substantially
2 equivalent to a native invertebrate lytic peptide.
- 1 **5.** A cell as recited in Claim 3, wherein said cell comprises a human cell.
- 1 **6.** A cell as recited in Claim 5, wherein said lytic peptide is substantially
2 equivalent to a native invertebrate lytic peptide.
- 1 **7.** A cell as recited in Claim 3, wherein said cell comprises a fetal donkey
2 dermal cell.
- 1 **8.** A cell as recited in Claim 3, wherein said cell comprises an embryonal stem
2 cell.
- 1 **9.** A cell as recited in Claim 3, wherein said cell comprises a hematopoietic
2 stem cell.
- 1 **10.** A cell as recited in Claim 9, wherein said cell comprises a human
2 hematopoietic stem cell.
- 1 **11.** A cell as recited in Claim 3, wherein said cell comprises a leukocyte.
- 1 **12.** A cell as recited in Claim 11, wherein said cell comprises a tumor infiltrating
2 lymphocyte, a neutrophil, a macrophage, or a cytotoxic lymphocyte.

1 **13.** A cell as recited in Claim 12, wherein said cell comprises a human tumor
2 infiltrating lymphocyte, a human neutrophil, a human macrophage, or a human cytotoxic
3 lymphocyte.

1 **14.** A cell as recited in Claim 13, wherein said cell comprises a human tumor
2 infiltrating lymphocyte.

1 **15.** A cell as recited in Claim 1, wherein said cell comprises a catfish cell.

1 **16.** A catfish comprising a cell as recited in Claim 15.

1 **17.** A cell as recited in Claim 1, wherein said cell comprises a chicken cell.

1 **18.** A chicken comprising a cell as recited in Claim 17.

1 **19.** A cell as recited in Claim 1, wherein said cell comprises a gene encoding at
2 least a 20-amino-acid-residue-portion of a peptide selected from the group consisting of a
3 cecropin peptide, a melittin peptide, a defensin peptide, a magainin peptide, a sarcotoxin
4 peptide, and analogs of said peptides.

1 **20.** A eukaryotic cell comprising a gene under the control of a promoter that is
2 exogenous to said cell, wherein said promoter is responsive to an inducer of an acute-
3 phase peptide or protein.

1 **21.** A cell as recited in Claim 20, wherein said promoter is substantially
2 equivalent to a native invertebrate promoter.

1 **22.** A cell as recited in Claim 20, wherein said cell comprises a vertebrate cell.

1 **23.** A cell as recited in Claim 22, wherein said promoter is substantially
2 equivalent to a native invertebrate promoter.

1 **24.** A cell as recited in Claim 22, wherein said cell comprises a mammalian cell.

1 **25.** A cell as recited in Claim 24, wherein said cell comprises a hematopoietic
2 stem cell.

1 **26.** A cell as recited in Claim 20, wherein said promoter is substantially
2 equivalent to a native promoter that controls the expression of a native lytic peptide.

1 **27.** A cell as recited in Claim 26, wherein said promoter comprises a promoter
2 selected from the group consisting of native promoters for a cecropin, native promoters
3 for a melittin, native promoters for a defensin, native promoters for a magainin, and
4 native promoters for a sarcotoxin.

1 **28.** A cell as recited in Claim 27, wherein said promoter comprises the native
2 cecropin B promoter.

1 **29.** A cell as recited in Claim 28, wherein said cell comprises a mammalian cell.

1 **30.** A cell as recited in Claim 29, wherein said cell comprises a hematopoietic
2 stem cell.

1 **31.** A cell as recited in Claim 27, wherein said cell comprises a mammalian cell.

1 **32.** A cell as recited in Claim 31, wherein said cell comprises a hematopoietic
2 stem cell.

1 **33.** A cell as recited in Claim 26, wherein said cell comprises a mammalian cell.

1 **34.** A cell as recited in Claim 33, wherein said cell comprises a hematopoietic
2 stem cell.

1 **35.** A vector for transforming a eukaryotic cell, wherein said vector comprises a
2 gene under the control of a promoter that is responsive to an inducer of an acute-phase
3 peptide or protein; and wherein said vector is adapted to insure that when a cell is
4 transformed by said vector, then both the gene and the promoter from said vector are fully
5 incorporated into the cell's genome.

1 **36.** A vector as recited in Claim 35, wherein said promoter is substantially
2 equivalent to a native invertebrate promoter.

1 **37.** A vector as recited in Claim 35, wherein said vector is adapted to transform
2 a vertebrate cell.

1 **38.** A vector as recited in Claim 37, wherein said promoter is substantially
2 equivalent to a native invertebrate promoter.

1 **39.** A vector as recited in Claim 37, wherein said vector is adapted to transform
2 a mammalian cell.

1 **40.** A vector as recited in Claim 35, wherein said promoter is substantially
2 equivalent to a native promoter that controls the expression of a native lytic peptide.

1 **41.** A vector as recited in Claim 40, wherein said promoter selected from the
2 group consisting of native promoters for a cecropin, native promoters for a melittin, native
3 promoters for a defensin, native promoters for a magainin, and native promoters for a
4 sarcotoxin.

1 **42.** A vector as recited in Claim 41, wherein said promoter comprises the native
2 cecropin B promoter.

1 **43.** A vector as recited in Claim 42, wherein said vector is adapted to transform
2 a mammalian cell.

1 **44.** A vector as recited in Claim 41, wherein said vector is adapted to transform
2 a mammalian cell.

1 **45.** A vector as recited in Claim 40, wherein said vector is adapted to transform
2 a mammalian cell.

1 **46.** A method for treating infection in a mammal, comprising the steps of:
2 (a) placing a transformed hematopoietic stem cell as recited in Claim 9 into the
3 mammal; and
4 (b) inducing expression of said gene, whereby the lytic peptide is expressed.

1 **47.** A method as recited in Claim 46, wherein the mammal treated is a human.

1 **48.** A method as recited in Claim 47, wherein the infection treated comprises a
2 human immunodeficiency virus infection.

1 **49.** A method as recited in Claim 46, wherein the infection treated comprises a
2 bacterial infection.

1 **50.** A method as recited in Claim 46, wherein the infection treated comprises a
2 viral infection.

1 **51.** A method as recited in Claim 50, wherein the infection treated comprises an
2 equine infectious anemia infection.

1 **52.** A method as recited in Claim 46, wherein the infection treated comprises a
2 protozoal infection.

1 **53.** A method as recited in Claim 46, wherein the infection treated comprises a
2 fungal infection.

1 **54.** A method for treating a tumor in a mammal, comprising the steps of:
2 (a) placing a cell as recited in Claim 11 into the mammal; and
3 (b) inducing expression of said gene, whereby the lytic peptide is expressed.

1 **55.** A method for treating a tumor in a mammal, comprising the steps of:
2 (a) placing a cell as recited in Claim 12 into the mammal; and
3 (b) inducing expression of said gene, whereby the lytic peptide is expressed.

1 **56.** A method for treating a tumor in a human, comprising the steps of:
2 (a) placing a cell as recited in Claim 13 into the human; and
3 (b) inducing expression of said gene, whereby the lytic peptide is expressed.

1 **57.** A method for treating a tumor in a human, comprising the steps of:
2 (a) placing a cell as recited in Claim 14 into the human; and
3 (b) inducing expression of said gene, whereby the lytic peptide is expressed.

1 **58.** A cell as recited in Claim 1, wherein said gene is under the control of a
2 promoter that is exogenous to said cell, and wherein said promoter is responsive to an
3 inducer of an acute-phase peptide or protein.

1 **59.** A cell as recited in Claim 2, wherein said gene is under the control of a
2 promoter that is exogenous to said cell, and wherein said promoter is responsive to an
3 inducer of an acute-phase peptide or protein.

1 **60.** A cell as recited in Claim 3, wherein said gene is under the control of a
2 promoter that is exogenous to said cell, and wherein said promoter is responsive to an
3 inducer of an acute-phase peptide or protein.

1 **61.** A cell as recited in Claim 4, wherein said gene is under the control of a
2 promoter that is exogenous to said cell, and wherein said promoter is responsive to an
3 inducer of an acute-phase peptide or protein.

1 **62.** A cell as recited in Claim 5, wherein said gene is under the control of a
2 promoter that is exogenous to said cell, and wherein said promoter is responsive to an
3 inducer of an acute-phase peptide or protein.

1 **63.** A cell as recited in Claim 6, wherein said gene is under the control of a
2 promoter that is exogenous to said cell, and wherein said promoter is responsive to an
3 inducer of an acute-phase peptide or protein.

1 **64.** A cell as recited in Claim 8, wherein said gene is under the control of a
2 promoter that is exogenous to said cell, and wherein said promoter is responsive to an
3 inducer of an acute-phase peptide or protein.

1 **65.** A cell as recited in Claim 9, wherein said gene is under the control of a
2 promoter that is exogenous to said cell, and wherein said promoter is responsive to an
3 inducer of an acute-phase peptide or protein.

1 **66.** A cell as recited in Claim 10, wherein said gene is under the control of a
2 promoter that is exogenous to said cell, and wherein said promoter is responsive to an
3 inducer of an acute-phase peptide or protein.

1 **67.** A cell as recited in Claim 11, wherein said gene is under the control of a
2 promoter that is exogenous to said cell, and wherein said promoter is responsive to an
3 inducer of an acute-phase peptide or protein.

1 **68.** A cell as recited in Claim 12, wherein said gene is under the control of a
2 promoter that is exogenous to said cell, and wherein said promoter is responsive to an
3 inducer of an acute-phase peptide or protein.

1 **69.** A cell as recited in Claim 13, wherein said gene is under the control of a
2 promoter that is exogenous to said cell, and wherein said promoter is responsive to an
3 inducer of an acute-phase peptide or protein.

1 **70.** A cell as recited in Claim 14, wherein said gene is under the control of a
2 promoter that is exogenous to said cell, and wherein said promoter is responsive to an
3 inducer of an acute-phase peptide or protein.

1 **71.** A cell as recited in Claim 15, wherein said gene is under the control of a
2 promoter that is exogenous to said cell, and wherein said promoter is responsive to an
3 inducer of an acute-phase peptide or protein.

1 **72.** A catfish as recited in Claim 16, wherein said gene is under the control of a
2 promoter that is exogenous to said cell, and wherein said promoter is responsive to an
3 inducer of an acute-phase peptide or protein.

1 **73.** A cell as recited in Claim 17, wherein said gene is under the control of a
2 promoter that is exogenous to said cell, and wherein said promoter is responsive to an
3 inducer of an acute-phase peptide or protein.

1 **74.** A chicken as recited in Claim 18, wherein said gene is under the control of a
2 promoter that is exogenous to said cell, and wherein said promoter is responsive to an
3 inducer of an acute-phase peptide or protein.

1 **75.** A cell as recited in Claim 19, wherein said gene is under the control of a
2 promoter that is exogenous to said cell, and wherein said promoter is responsive to an
3 inducer of an acute-phase peptide or protein.

1 **76.** A vector for transforming an exogenous gene into a eukaryotic cell,
2 comprising:

3 (a) a gene encoding a transposase;

4 (b) two transposon insertion sequences;

5 (c) said exogenous gene, wherein said exogenous gene is between the two said
6 insertion sequences; and

7 (d) a promoter that is adapted to cause the transcription of said transposase;

8 wherein one of said insertion sequences is located between said transposase gene and said
9 exogenous gene; and wherein the transposase is adapted to excise from said vector a
10 fragment comprising the two said insertion sequences and the portion of said vector
11 between the two said insertion sequences, and to insert the excised fragment into a
12 chromosome of a eukaryotic cell.

1 **77.** A vector as recited in Claim 76, wherein said promoter is inducible by an
2 inducing stimulus to cause the transcription of said transposase.

1 **78.** A vector as recited in Claim 77, wherein said vector comprises plasmid
2 pCEP.

1 **79.** A vector as recited in Claim 78, wherein said exogenous gene comprises a
2 gene encoding native cecropin B.

1 **80.** A vector as recited in Claim 78, wherein said exogenous gene comprises a
2 gene that is not a gene encoding native cecropin B.

1 **81.** A cell comprising the *E. coli* strain with ATCC accession number 69342; or
2 a cell comprising a mutant, recombinant, or genetically engineered derivative of the *E.*
3 *coli* strain with ATCC accession number 69342; or a cell that is the progeny of any of
4 these cells; wherein said cell comprises a plasmid that expresses the Tn10-transposase-
5 based transformation activity of the plasmid pCEP.

1 **82.** A cell as recited in Claim 81, wherein said cell comprises the *E. coli* strain
2 with ATCC accession number 69342.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/07456

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A01K 67/00; A01N 63/00; A61K 37/00; C12N 1/20, 5/00, 15/00; C12P 21/06

US CL : 424/93B; 435/69.1, 172.1, 172.3, 240.1, 252.3, 252.33, 320.1; 800/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93B; 435/69.1, 172.1, 172.3, 240.1, 252.3, 252.33, 320.1; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 90/12866 (JAYNES) 01 November 1990, see the entire document.	1-75
Y	WO, A, 89/00199 (JAYNES ET AL) 12 January 1989, see the entire document.	1-75
Y	Nature, Volume 357, issued 11 June 1992, A.D. Miller, "Human gene therapy comes of age", pages 455-460, see the entire document.	46-57
Y	EMBO Journal, Volume 9, issued 1990, P. Kylsten et al, "The cecropin locus in Drosophila; a compact gene cluster involved in the response to infection", pages 217-224, see the entire document.	1-75



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

31 AUGUST 1994

Date of mailing of the international search report

OCT 13 1994

Name and mailing address of the ISA/US
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07456

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EMBO Journal, Volume 11, No. 4, issued 1992, J-M. Reichhart et al, " Insect immunity: developmental and inducible activity of the Drosophila dipteracin promoter", pages 1469-1477, see the entire document.	20-45
X	EMBO Journal, Volume 6, No. 13, issued 1987, G. Ciliberto et al, "Inducible and tissue-specific expression of human C-reactive protein in transgenic mice", pages 4017-4022, see the entire document.	20, 22, 24, 35, 37, 39
Y	US, A, 5,102,797 (TUCKER ET AL) 07 April 1992, see the entire document.	76-82
Y	Gene, Volume 32, No. 3, issued 1984, J.C. Way et al, "New Tn10 derivatives for transposon mutagenesis and for construction of lacZ operon fusions by transposition", pages 369-379, see the entire document.	76-82

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07456**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, CHEMICAL ABSTRACTS, EMBASE, DERWENT BIOTECHNOLOGY ABSTRACTS

search terms: vector, transformation, transposon, transposase, cecropin, melittin, defensin, magainin, sarcotoxin, lytic peptide, catfish, chicken, donkey, human, acute phase, promoter

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-75, drawn to transformed animal cells, methods for their use, and transformation vectors, classified in class 435, subclass 240.2.

Group II, claim(s) 76-82, drawn to transformation vectors comprising a transposase-encoding sequence, classified in class 435, subclass 320.1.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The vectors, transformed cells and methods of I are not required for the production or use of the vectors of II. The vectors of II are not required for the production or use of the vectors, cells and methods of I. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.